sort of albeit transient side effect following immunization.

So, these are quite reactogenic vaccines, and in the groups of people who are in the UK with this vaccine, it is not infrequent for people to be sick for a day or two following booster immunizations.

So, that is killed whole cell vaccines. What about live attenuated vaccines? They have never really been used in the West, in the U.S. or in Europe, but they have been used in the former Soviet Union, quite extensively actually, and in some of the French Colonies like Madagascar.

It is quite a high immunizing dose, 6 million CFU, and just to relate the way these vaccines work in comparison to the killed whole cell vaccines, after immunization, what you can demonstrate is sera from immunized animals or individuals, that should work in the Mouse Neutralization test, and the Mouse Protection Index is typically less than 10 after immunization, so it is kind of consistent with this vision, this

picture that a Mouse Protection Index of less than 10 is indicative of protection in that passive transfer model.

There are a number of these EV vaccines that differ very slightly. They all have the same heritage. They are all pigmentation mutants actually, so in contrast to wild type strains of Yersinia pestis, which become pigmented when they are grown on certain agars, like Congo Red agar, these EV series strains are nonpigmented, and it is not fully clarified why they are not pigmented. It is almost certain that they have a number of mutations in the so-called pgm locus, and possibly that affects their ability to acquire iron in the way that Bob Perry talked about this morning.

The precise reasons for attenuation of the EV series vaccines at a molecular level is not known. Very reactogenic. In one study in 1970, in the U.S., in human volunteers, it was reported remarkably that 100 percent of people who were immunized with the ED vaccine developed severe systemic reactions.

Some individuals in Russia, who were immunized with the EV vaccines required hospitalization, and it is quite frequent to have severe local reactions surrounding the site of immunization. So, these vaccines are even more reactogenic than the killed whole cell vaccines.

But they are effective, and they are effective apparently against both subcutaneous and inhalation challenges. So, in this experiment, animals were immunized via the intramuscular route with ED76, and then challenged by the inhalation route. All of the control animals died, but all of the EV-immunized animals are protected.

So, in contrast to the killed whole cell vaccines, these live attenuated vaccines do appear to protect quite well against an inhalation challenge.

So, in summary, killed whole cell vaccines, not very good, don't protect against pneumonic plague. Live vaccines, like the ED series, do protect against pneumonic plague, but they are highly reactogenic, and they have never

really been accepted at least in the West.

So, what about an improved vaccine?

A number of approaches one might use to derive an improved vaccine, one might try and derive a live attenuated mutant to replace the ED76 vaccine, a safe live attenuated mutant, or one might try and identify the important protective components on Yersinia pestis and put those in some sort of subunit or make a DNA vaccine.

Starting off with live attenuated mutants, we spent quite a lot of time pool matching, trying to devise live attenuated mutants of Yersinia pestis, and our initial attempts were not particularly successful.

Although we can derive mutants which are attenuated in the murine model of disease, they are nowhere near attenuated enough, like the PhoP mutant, 75-fold attenuated, that is nowhere near attenuated enough for this kind of mutant to be considered as a live attenuated vaccine.

But more recently, there have been some successes. For example, a group in Israel recently

reported that they had isolated a pcm mutant of Yersinia pestis, which was over 10 million-fold attenuated in the murine model of disease.

The map pcm mutant does look like a possible live attenuated mutant vaccine, so this is a comparison of the way in which the pcm mutant on the ED76 strain perform in the murine model of disease. These are responses developed to F1 antigens, so these are F1 antibody responses induced by the pcm mutant and by the ED76 strain, V-antibody and level of protection.

The pcm mutant performs much, much better than the ED76 strain by any of these criteria that are compared in this graph. So, maybe there is a suggestion that some live attenuated mutants can be devised which have improved performance compared to the ED76 strain, but whether these kind of mutants will ever be acceptable for use in humans, I guess is a subject that is open to debate.

Subunit vaccines, we have looked at a whole range of subunits, and I know other people have, like Sue Straley, and so on, have looked at

various components of the type III system as candidates to go into some subunit vaccine.

To date, the only subunits that have been identified that provide good levels of protection, at least in the murine model of disease, are the F1 antigen and the V antigen. We can actually produce these proteins relatively easily using recombinant DNA technology, so to make the F1 antigen, we just transfer the entire F1 operon into E. coli, and that directs synthesis and export and assembly of F1 antigen on the surface of E. coli in much the same way as it would on the surface of Yersinia pestis, and you can harvest F1 antigen quite easily from the surface of the bacteria.

V antigen can be expressed very easily as a GST-fusion, fusion with a carrier protein like glutathione S-transferase, and in the system we use to generate V antigen, you cleave the V from the carrier using PreScission protease. So, we generate what is very close to an authentic N-terminus of the protein.

These individual subunits work very well

as protective antigens. This is experiments in the mice model of disease, so these are challenge doses increasing from 10^5 up to 10^9 CFU of Yersinia pestis. This is actually given by the subcutaneous route of challenge. These are control mice, so they will die at any of the challenge doses that we have tested.

These are mice that are immunized with F1 antigen, and they are protected against lower challenge doses, partially protected against lower challenge doses, but at these very high challenge doses, we see defeat of protection, similarly with V antigen, defeat of protection at very high challenge doses, but when these two components are formulated together, what we end up with is a vaccine that appears to provide very, very high levels of protection at least against the subcutaneous challenge with fully virulent Yersinia pestis.

Not only does it protect against a subcutaneous challenge, it protects very well against an inhalation challenge. So, again, this

is the murine model of disease, mice challenged by the injected route subcutaneously or by the inhalation route.

These are mice that have been immunized with one of the killed whole cell vaccines just for comparison. These are mice that have been immunized with recombinant F1 and V vaccine that we have devised, and as you can see, we can solidly protect these animals against either subcutaneous or an inhalation challenge.

We can demonstrate that protection against a range of different strains of Yersinia pestis including the F1-negative Java 9 strain.

So, that vaccine has been formulated as a two-dose injectable vaccine, and the current immunization schedule involves giving a dose on day 1 and a dose on day 21, and it is projected that it will involve somewhere around 40 micrograms of F1 and 40 micrograms of V antigen.

I guess one of the important questions, one of the questions that has come up from this morning, is that although people have looked for

additional protective antigens that might protect against plague, what is the evidence that there might be additional protective antigens.

This is an experiment we did very recently with a PYV cured strain of Yersinia pseudotuberculosis. It was also actually a dam mutant of Yersinia pseudotuberculosis, but maybe that is not too significant.

In this study, what we did was immunized mice either orally or intravenously with this PYV cured strain of Yersinia pseudotuberculosis and then challenged them with Yersinia pestis, and remarkably, you can protect pretty well after either oral or intravenous immunization with this mutant, and certainly, to us, that suggests there must be other protective antigens out there, but presumably are co-displayed by Yersinia pseudotuberculosis that are just waiting to be discovered. So, I am sure there are additional protective antigens out there.

What I would like to do finally for the next five minutes or so is just talk about the

prospects for third generation vaccines because maybe the vaccine that we are looking at for the moment, that we are developing at the moment, the F1-V vaccine is just one step towards an ideal vaccine against plague.

One of the requirements may be of a third generation vaccine is it can be given non-invasively, hopefully orally, as a single-dose vaccine, and one of the technologies we have been looking at is to transfer some of these protective antigen genes into Salmonella typhi, and in this experiment, what we did was transferred the gene clustering coding the F1 antigen into Salmonella typhi BRD1116. This is an aroA, aroC, htrA mutant. So, this is the same strain that is currently proposed as live, orally delivered typhoid vaccine.

There are typhoid bacteria expressing F1 antigen on the surface, so they actually make F1 antigen, they express it on the surface, and you can demonstrate expression of F1 antigen in macrophages infected with this recombinant Salmonella typhi, and you can demonstrate the

induction of protective responses in the appropriate model of disease.

This is the intranasal immunization model, so in this experiment, what we did is immunize the mice intranasally with Salmonella typhi expressing F1 antigen on the surface, and those mice are reasonably well protected, around 70 percent protected against subcutaneous challenge with 100 MLD of Yersinia pestis.

So, there is certainly a suggestion that we can devise single-dose, non-invasive, delivered orally or intranasally delivered vaccines.

Naked DNA vaccines are another possibility for future third generation vaccines, and there are some various reports out there actually indicating that naked DNA vaccines, which encode the F1- or V antigens are effective, that they induce protective responses against plague, but the problem is at the moment we need to give multiple doses of those naked DNA vaccines.

Often you need to use them as prime-boost strategies, and to me, it is not overly apparent

what advantages this type of vaccine have over subunit vaccine containing just the proteins you are interested in.

So, in summary, what I hope you have taken away from my presentation this morning, the kind of key messages are that the existing killed whole cell or live attenuated vaccines have significant limitations both with respect to their ability to protect against pneumonic plague, their reactogenicity, and the ease with which they would be or could be licensed in for use in humans.

Improved live attenuated vaccines do appear to be feasible, and there is that demonstration of proof of principle with the pcm mutant that I just talked about, but I guess there is always going to be a question about whether we are going to accept that type of mutant for large-scale immunization of human populations.

There are some suggestions that subunit vaccines, particularly based on the F1- and V antigens at the moment appear to be effective and appear to be safe, but there may well be additional

protective antigens out there waiting to be discovered and waiting to be formulated into an improved third generation, fourth generation plague vaccine.

Finally, there is some evidence indicating that orally or intranasally delivered vaccines against plague might become a realizable prospect as a third generation vaccine in the future.

Finally, just a list of collaborators. Most of the people who have been involved in this work have been located at Porton Down, but we have some very good collaborations with the London School in London, very good collaborations with our Swedish colleagues at the National Defense Research establishment in Sweden, and finally, some links with the University of Umea.

Thank you very much. I would be very happy to answer any questions.

[Applause.]

DR. QUINN: We have got about five minutes for questions.

DR. MIZEL: Steve Mizel, Wake Forest.

Do you have any evidence with your intranasal immunizations of any kind of neurotoxicity as seen with several other vaccines?

DR. TITBALL: We have not seen that with the recombinant Salmonella typhi, but I guess the intranasal immunization model is really just a model for all immunization in humans with Salmonella typhi, so maybe it is not necessarily the most meaningful as to whether you would see any neurological consequences.

We have actually, Di has done quite a lot of work giving purified F1- and V antigens intranasally in various microencapsulated formulations, and we have never seen any adverse side effects which indicate neurotoxicity when given by that route.

DR. NATARO: Jim Nataro, University of Maryland.

Several groups have proposed using attenuated pseudotuberculosis or enterocolitica, which obviously have some real advantages. You mentioned one series of studies. But there is

obviously a very high rate of postinfectious sequelae with those infections, arthritis, and even amyloidosis.

I am not familiar with that in pestis, but do you want to comment on whether that is being looked at, at all, or whether those vaccines are impeded?

DR. TITBALL: You are thinking about in people you could immunize with the live attenuated EV76 strain, because most people who are infected with Yersinia pestis, a reasonable proportion go on to die. I guess of those that recover, I am not aware there is any indication of any kind of arthritic complications of sequelae in those populations.

Similarly, I am not aware of any reported indications in Russian populations that have been immunized, but in some of those populations, it is kind of questionable whether those issues would have been recorded appropriately.

DR. NATARO: But do we assume that enterocolitica and pseudotuberculosis are dead as

far as vaccine candidates, because of the risk of postinfectious sequelae?

DR. TITBALL: I think it depends on which serotype of enterocolitica or pseudo-TB you pick. I don't know. No, I wouldn't say they were dead. I don't know whether anybody else wants to comment.

DR. SMILEY: Steve Smiley from Trudeau Institute.

As a follow-on to that question, so with the plasmid cured pseudotuberculosis, the protection you see there, do you know whether that is antibody mediated, can it be transferred?

DR. TITBALL: I have no idea. Those are all really important experiments that need to be carried out.

DR. : In your studies, you mentioned the challenge with a different type of modality, such as subcutaneous, intranasal, and aerosol. So, from your experience, do you think intranasal could in some way reflect pneumonic model?

DR. TITBALL: That is a very good

question. I think the preference is always to carry out an inhalation challenge, you know, if you have that capability, and we would not substitute an inhalation challenge or vice versa, but there is some evidence that actually, intranasal challenge does result in a disease which is very similar to that, that you see after inhalational challenge.

I guess there hasn't been enough detailed histopathology carried out to actually compare the disease in detail after challenge by those two different routes.

DR. : Because that would be very important to actually, intranasal would be much better controlled than the inhalation.

DR. TITBALL: Maybe, but I mean cytodeposition is always going to be slightly different. It depends on whether you are actually talking the deep lung or the upper respiratory tract.

DR. : Right, but actually, most of the respiratory is in upper respiratory infection.

DR. TITBALL: Right, but probably not after exposure to Yersinia pestis used a biowarfare agent. It is much more likely to be a lower respiratory tract which is targeted.

DR. QUINN: We have time for one last question.

DR. : The question I have concerns topics that came up earlier. This was a beautiful synthesis of what you can get with a vaccine.

Richard, what I want to know is did you try that actually at Porton Down on laboratory workers, that vaccine, and what do you think about their concerns about F1 that were raised here earlier and the immunosuppressive role of LcrV, and how do you feel about it?

DR. TITBALL: Those are good questions. I think Di is going to talk about some of the clinical trials we have carried out with this vaccine. Our F1 and V vaccine has been into people, and there are no obvious indications of adverse side effects.

Clearly, one of the reasons for including F1 and V in any future vaccine has to do with this issue of possible virulent strains that lack F1 antigen on the surface, and I guess the issue about the possible immunosuppressive properties of V, you know, it is probably best, though, by considering in those experiments where immunomodulatory properties have been reported, the V antigen has been given repeatedly at daily intervals in quite large doses.

It has not, to my knowledge, been given as a single low-dose cell-purified protein.

DR. : Let me just ask you, what do you think of the 2 "Animal Rule" in terms of plate testing that is proposed to the U.S., because I think you probably work in different coordinates than EU.

DR. TITBALL: Not necessarily actually. I mean it would be the same kind of considerations for us in the UK that we would need to demonstrate efficacy in at least two animal species.

DR. QUINN: We will introduce our next

speaker, Dr. Diane Williamson from Defence Science and Technology Laboratory, Porton Down, who will be talking to us about the role of antibodies and cell-mediated immunity in protection.

The Role of Antibodies and Cell-Mediated Immunity in Conferring Protection Against Plague

Dr. Diane Williamson

DR. WILLIAMSON: Thank you. Good morning and I would like to start by thanking the organizers for inviting me to participate in this workshop. It is a great privilege to be here.

I just want to point out that my hardcopy of my presentation is actually on your supplements in your binders. I reordered the size in order to try and address some of the questions that I thought the panel might be concerned with tomorrow and also to try and prevent duplication of some of the subsequent speakers talking about small animal models, so if you follow my presentation, if you would like to follow the supplement rather than the bound-in copy.

I am going to try to cover the role of

antibodies and cell-mediated immunity in conferring protection against plague. Of course, you have heard that plague is predominantly an extracellular infection with intracellular phases.

So, what does this mean in terms of the immune response? Protection against plague will depend on countering the bacterium and its virulence factors. We have heard already a lot about the virulence factors that this organism is able to produce.

The host, in order to protect itself, will need to induce an appropriate immune response or will need to be induced to produce an appropriate immune response by vaccination, and we are going to talk about antibody and cell-mediated immunity in that context.

Of course, because this is a serious human pathogen, and because field trials showing efficacy are not going to be easily achieved, we need to depend very heavily on animal models to elucidate these protective immune responses.

I want to talk this morning a little bit

about the data that we have gathered so far in mouse, guinea pig, beginning to gather in the marmoset with the small nonhuman primate model, and in macaque, and also some immunogenicity data that we have gathered so far in a safety trial of vaccine in man.

So, just starting with the mouse, what we have here is a very early study where we showed that F1 and V in combination were protective in the mouse model, and this is a BALB/c inbred mouse model, against a human fatal isolate of plague, and they conferred the same level of protection as to live attenuated ED76 vaccine, and, by comparison, the killed whole cell vaccine was defeated against this very high, 10^9 subcutaneous challenge.

So, antibody is probably very important in protection. What I want to do now is just characterize what we know about the kinetics of antibody production in our animal models, quantity of antibody produced. I want to look at the similarities between the animal models, and then try to relate the antibody characteristics that we

have observed to protective efficacy, and then look at the rationale for extrapolation from the animal models to man.

Just looking at the kinetics first, we have looked previously at the antibody response to the F1 plus V combined vaccine in four different haplotypes of mice, and you can see that from this kinetic study where mice were immunized at day naught and at day 21, that antibody response started to rise very fast and peaked at about a week after the second dose of vaccine. These animals were then boosted later on, and cell-mediated antibody was followed right out for several months.

But the take-home message from this slide is that although we have four different haplotypes of mouse here, they are all responding in a very similar way with their antibody kinetics.

When we challenged these mice at day 80, we saw some subtle differences in protection against subcut challenge. This is a very high challenge level. It is a very virulent strain of

plague, however, and I don't think these really are very significant differences.

When we challenged the mice by the aerosol route, we saw solid protection at this time point. So, in these inbred strains of mice, haplotype doesn't seem to have very great an influence on levels of protection achievable.

We also looked at gender within these haplotypes and compared male and female mice responses, and saw little difference there either.

We went on to select the BALB/c mouse for most of our other subsequent studies and here I am showing you a dose response curve in the BALB/c mouse where we immunized with decreasing concentrations of the F1 and V subunits and challenged the mice with 10^7 CFU subcutaneously, or 10^5, and you can see that the minimum protective dose against the 10^5 CFU is around the 1 microgram mark, and the minimum protective dose against the 10^7 CFU is around the 5 microgram mark of vaccine.

We were able to correlate the predominant IgG subcuts or haplotype IgG1 with protection in

the mouse, and showed that as you decreased the dose, you lost IgG1, and that relationship correlated significantly.

We also looked at the protective efficacy of vaccine in outbred mouse strain, and we, at Porton, have an outbred closed colony, outbred mouse strain, which we call the Porton mouse. It is a very stable strain. We immunized these mice with the F1 plus V combination and challenged them at day 60 of the two immunizing doses with plague by the aerosol route, and we showed very solid protection against 100 LD50 of plague by the aerosol route.

We also actually escalated the immunizing dose up to 75 micrograms and gave it on a single occasion in this last part here, and challenged these mice by the aerosol route and showed that we could protect them against 10^4 LD50, which is the maximum protection we have shown against the challenge in the mouse model to date.

Having escalated the vaccine dose up to 75 micrograms, we did some more exploratory work where

we looked at the increasing doses of the vaccine given very soon prior to challenge, and here, we have some data which just shows, in the BALB/c mouse model against an aerosol challenge of 300 lethal doses, but even giving the vaccine three days prior to challenge, one can get some protective effect, and giving it six days at 25 micrograms of each subunit, one can get full protection.

So, this is quite encouraging data, may translate to the use of vaccine postexposure if you need six days to achieve protective immunity, that might set your time frame for postexposure therapy.

Moving on now to the guinea pig. We have done some limited work in the guinea pig. The guinea pig, we do not find to be a very good model of plague infection. The plague infection seems to be very chronic in the guinea pig, unlike the mouse where you have an acute infection, the guinea pig seems there is a very chronic infection.

When we looked at antibody responses in our guinea pigs, in our immunized guinea pigs, at

time of challenge, we saw very variable responses to the F1 antigen, and much more consistent responses to the V antigen.

When we challenged these immunized animals, we managed to achieve full protection against an injected challenge of 10^5 lethal doses, 10^5 CFU, and then partial protection beyond that.

But we are not planning to pursue the guinea pig too much further as a model because of the difficulties, and you can see that in survivors here, we had very protracted time to death as the infection became very chronic.

We have done some work in cynomolgus macaques, and I am just going to describe to you an immunogenicity study where we looked at ascending dose levels of vaccine in this range in male and female cynomolgus macaques immunized on two occasions.

Here, we have typical antibody response. This is to the V antigen in these animals. I have shown just the 10 microgram, the response to the 10-microgram dose group and the 40-microgram dose

group just for comparison, and you can see that animals were immunized at week naught and at week 3, we get some very nice secondary antibodies form to the booster dose in the green and red bar.

The yellow line here represents animals that were given a single immunizing dose at the 40-microgram dose level of the vaccine, and you can see that they responded reasonably well, but, of course, didn't develop the secondary immune response.

Moving on now to observations of antibody responses in man, we have done a preliminary Phase I safety study in Europe in 32 individuals given the vaccine in the same dose range as used in the macaque study, alhydrogel adjuvant, and we have looked at safety and found absolutely no safety concerns with this vaccine.

We looked at some cytokine readouts, for instance, IL-6, and saw no change in vaccinees in that IL-6 level, and additionally, we were able to do some immunogenicity work with serum from the volunteers, and what we found was that when we

immunized in this dose range, we got this kind of pattern of antibody response. All individuals responded to either of the antigens. Some did not respond to F1, some did not respond to V, but generally, at the 40 microgram dose level, we had complete response to both antigens.

You can see a dose response effect here with increasing agglutinine [?] titers with dose level.

Just turning now to antibody functionality, what I have talked about so far really are observations on kinetics and quantity of antibody, but what does that mean in terms of antibody functionality?

We can look at neutralizing antibody by competitive ELISA, we can actually look at the inhibition of the cytotoxic effect of V antigen as expressed in pseudotuberculosis construct in vitro, and we can look at passive transfer, and I just want to quickly run through the data we have to date in this context.

We have developed, at the research level,

a competitive ELISA for V antigen, and this ELISA depends on coating with V antigen, and then introducing the monoclonal antibody 7.3, which we have previously shown to be protective against plague challenge when given by passive transfer in the mouse.

That monoclonal antibody binds to the antigen and we start with 100 percent binding of that, and then we introduced vaccinee serum at various dilutions. When you introduce macaque serum in this case, 1 in 80 dilution, you begin to see competition with the mouse monoclonal antibodies binding to V and some loss of mouse antibody signal here.

As you increase the concentration of your vaccinee serum, you can see that you get complete inhibition of binding of the mouse antibody to V. Now, we have done that for macaque serum, and we have also used the same ELISA to evaluate our antibody responses in people receiving the vaccine at the highest dose level tested.

What we found was that all individuals at

this 40 microgram dose level had neutralizing antibody for the V antigen and the serum, and also that neutralizing antibody correlated with total IgG, significant correlation with total IgG that those individuals were producing.

Just moving on to in vitro cytotoxicity, there is an assay that we and others are using where you can express V antigen from pestis, from pseudotuberculosis, and that construct is [inaudible] for macrophages in vitro.

You can therefore use this assay to look for inhibition of the cytotoxic effect with your vaccinee serum, and at the moment, this assay in our hands is a qualitative assay. Here, we have some readouts from the assay.

Here, we have macrophage cells in culture which are uninfected and green cells glow green, live cells glow green, and dead cells glow red. So, you can see that they are predominantly live here.

When you introduce the pseudotuberculosis V expressing strain, together with the protective

monoclonal antibody, you get protection against the effects of V, and you get a predominantly live culture.

Here, we have, though, a culture where we have introduced macaque serum taken on day 1 of a macaque immunization protocol, so you wouldn't expect antibody to the antigen in this serum, and indeed we get almost full killing of the culture.

When you take serum from that same macaque at week 10 schedule, you can see that it now has developed neutralizing antibodies to V antigen, and protecting the culture from killing in this assay.

Similarly, when we took serum from macaques that had been immunized, and we took serum at week 6 or week 10, we got similar protective effects.

So, this assay is giving us qualitative positive readout and showing that there are neutralizing antibodies in sera from these animals.

Just turning now to passive transfer, Dr. Titball mentioned passive transfer as a means of evaluating the vaccine in his presentation. We

have done a lot of passive transfer from the species into the mouse at Porton, and what we have found is that when BALB/c mice are immunized with the vaccine, and this is on 3 occasions, and then the serum is taken and transferred into SCID/Bge mice, and these are severe combined immunodeficient mice with the beige mutation, they have no functional immune system.

We can protect the recipient mice against challenge by the subcutaneous route and by the aerosol route. You will note that there is some breakthrough at the end of this 10-day assay for both challenge models, but this is probably attributed to the half-life of the passively delivered serum decaying and one then gets breakthrough. We now cap this assay or limit this assay to a 10-day assay.

We have done a similar kind of exercise with IgG purified from immunized guinea pig serum, and here we used IgG at two dose levels purified from the guinea pig serum, and got very similar data.

This IgG has been passively transferred into mice, and the mice have been challenged by the subcutaneous route, and guinea pigs given the F1 and V vaccine, their IgG fully protect mice. We were able to fully protect mice with IgG taken from guinea pigs given the existing plague vaccine, which we have supplemented with V antigen to immunize guinea pigs with.

Similarly, we have transferred IgG purified from immune macaque serum into mice and shown that it can fully protect groups of mice.

Now, what we have here are IgG at the 100-microgram dose level of IgG taken from macaques and mice at the different dose level of the vaccine that I showed you before, 5, 10, 20, and 40, and the single dose 40, and you can that IgG taken from those groups at all the dose levels of the vaccine was protected in the 10-day assay in the mice. So, we are able to transfer protective immunity with antibody.

Finally, in the human model, we have taken serum from donors in the 40-microgram dose level

group, human donors, and transferred their serum into mice and shown either full or partial protection of the mice, and we were able to correlate the protective immunity transferred with the IgG content to the donor serum, and there is a significant correlation there.

So, passive transfer would seem to be a useful method of evaluating serological protective immunity, but what we have found actually is that these assays are very relevant very early in the schedule, up to day 28 or so of the immunization schedule, and people responding with maximum serological antibody, but beyond that, some of the correlations start to fall away.

So, what we need to look at also is the cell-mediated immune response, and this is a rather harder function to assess. We have done some T-cell recall responses in BALB/c mice at 8 months post their original immunization, and shown that they do have significant recall responses, particularly for the V antigen.

So, what else can we do, what else do we

do to look at cell-mediated immunity? Cell-mediated immunity is undoubtedly an influence. The IgG subclass profile that we are seeing in these F1 plus V vaccinated individuals from all species indicates that what we are inducing is predominantly a Th2 response, and that is not a surprise, because we adjuvanted our vaccine with alhydrogel, and flow cytometry analysis in species that we have looked at, mouse, macaque, and man does indicate that what we have here is a CD4-positive memory response, which could be either Th2 or Th1.

But we also have some evidence from mouse models that a Th1 response to challenge is also essential to clear the infection, so although our vaccine is inducing predominantly Th2 response, the vaccinees are able to mount a Th1 response, they are able to mount a Th1 response, and that is essential to clear the infection.

Just to summarize very quickly a lot of work that we have done in genetic knockout models, we have looked in genetic mouse models which have a

targeted gene deletion in the STAT 6 pathway, and these animals are not able to mount a full Th2 response, but they do have an intact Th1 response.

Conversely, we have looked at targeted gene deletions in the STAT 4 pathway where these animals in a C27 background cannot mount a Th1 response, but do have a full Th2 response.

What we found with these animals was that a reduced vaccine efficacy occurred in STAT 4 knockout mice, and this correlates with absence of CD4 Th1 response, so that when we immunized these mice in the usual way, and challenged them at day 60 with plague, by the subcutaneous route, we saw breakthrough first in the STAT 4 knockout mice, and STAT breakthrough, but as we increased that challenge dose, we saw full breakthrough.

So, these animals are able to produce, mount a Th2 antibody, full antibody response, but cannot mount a Th1 response, and they are susceptible.

So, it looks as if both Th2 and Th1 responses are required for full protection against

plague, and when we looked a little bit further into this, we collected splenocytes from cohorts of these different strains that we have used in this experiment and re-presented them in vitro with the F1 and V antigens, and showed that whether they have been vaccinated, in the blue bar, or not, in the red bar, these strains were able to produce interferon-gamma in response to resubmission with the F1 and V antigens in vitro, but the STAT 4 models were not, as we expected, and it would seem, therefore, that the deficiency in protection in the STAT 4 mice can be related to lack of a Th1 response.

Just very finally, we are doing a lot of work at the moment looking at trying to map T cell epitopes in both F1 and V antigens, and we have nearly complete maps of the murine T cell epitopes in the V and the F1 antigens.

Now, what we hope next to do is to start to ascribe some function to those epitopes and then maybe to use peptides that represent those epitopes for which we have ascribed function as better

targets for assessing cell-mediated immunity in man as we proceed into our clinical trials.

So, therefore, in summary, we have shown an antibody response in all species that we have looked at with the F1 and V antigen, and this appears to be fairly conserved as we present these antigens in the alhydrogel formulation across species. Functionality is quantifiable in the tests that I have described, for instance, competitive ELISA, the inhibition of cytotoxicity, and in passive transfer.

That is certainly a mixed Th2/Th1 response is required to clear infection, and it would appear that presenting the F1 and V antigens in alhydrogel will induce cross-prime to both those responses. Cell-mediated immunity is the better black box at the moment. We know it is quantifiable by in vitro proliferation type assays. Perhaps by defining the T cell epitopes further, we will be able to provide improved targets to assess cell-mediated immunity more effectively.

Then, finally, there have been a number of

people, very many people involved in this project over the years at Porton. I have tried to list them all. We also have very good work ongoing currently, headed by our project office at Porton, in transitioning the vaccine from research into development, and that this is staffed by people with regulatory and clinical experience.

Of course, we are also indebted to Avecia Biotechnology, who in recent years have been manufacturing the vaccine for us, and we have a very good relationship with Newcastle University.

Thank you. [Applause.]

DR. QUINN: Thank you, Di.

We have time for some questions.

DR. FROTHINGHAM: Rich Frothingham, Duke University.

That was a very exciting lecture and I am delighted to hear how quickly this work has moved along with this combined recombinant subunit vaccine.

You mentioned that T cell epitopes have now been mapped for F1 and V. Is that information

available?

DR. WILLIAMSON: Not yet. We are about to submit some of that data, but it is just being completed at the moment.

DR. STRALEY: Sue Straley, University of Kentucky.

I am curious, in relation to, say, the development of monoclonal cocktails, whether anyone has looked at a difference in efficacy of different isotypes, IgG2A versus IgG1.

DR. WILLIAMSON: Well, we did actually attempt to do that some years ago, but working in the mouse, isolating these isotypes from the mouse in quantity was not easy. We actually attempted that experiment, but really were not able to proceed because we didn't have enough of the polyclonal-derived isotapes.

Strangely enough, many of the monoclonals that we have are IgG1 and difficult, but I am very keen to find any monoclonals out there, IgG2A or 2B biased, that would be of great interest.

DR. STRALEY: So, your 7.3 is an IgG1.

DR. WILLIAMSON: Yes.

DR. FERRIERI: Pat Ferrieri, University of Minnesota Medical School.

Is there consistency among different laboratories in the aerosol challenge, and specifically, my question is, are you pumping bacteria into a chamber, or on the other hand, are you dripping it into the nose and having them inhale it?

DR. WILLIAMSON: Right. We have had extensive interaction with USAMRIID in establishing the aerosol model. We actually aerosolized with Henderson apparatus or Collison [ph] spray, and we conditioned the aerosol appropriately in terms of humidity and temperature, so we got live bacteria deposited into the deep lung in our mouse model.

The animals are conscious when we do this, so we have a lot of experience of aerosolizing, and think we can keep the organisms viable.

DR. SCHNEEWIND: Olaf Schneewind, University of Chicago.

The query that I have has to do with the

publication of Jurgen Heesemann, who used isolated macrophages and showed that the LcrV stimulates an IL-10 release. Is that an assay that you feel should be included in studying the antibody response against LcrV, and, if so, would that be useful for mirroring [?] human macrophages?

DR. WILLIAMSON: We actually have some work ongoing with Heesemann's group. I have supplied him with the antigen to look at exactly that. Yes, certainly, we are to see what comes out of that collaboration.

DR. SCHNEEWIND: In this regard, I was interested in the human studies that you are doing, and you said that you had looked at a cytokine response for IL-6.

DR. WILLIAMSON: Yes.

DR. SCHNEEWIND: What time after infection do you study this?

DR. WILLIAMSON: We looked, not infection after immunization. We looked at the recall points for the volunteers two days after immunization regularly and saw no change in IL-6.

DR. SCHNEEWIND: And the studies in animal suggest that these changes occur within the first 24 hours for IL-10 and IL-6.

DR. WILLIAMSON: Yes. This is probably the logistics of running a clinical trial, one can't have volunteers coming back every day, but really I suppose in terms of immunosuppression, we are interested in whether there might be a long-term immunosuppressive effect of vaccine, so that is why we chose those time points. We couldn't see anything.

DR. QUINN: Last question.

DR. MORRIS: Stephen Morris.

I was wondering, USAMRIID has also used the African Green monkey as a challenge model. Could you comment on the considerations that went into your decision to use the cynomolgus macaque as opposed to that particular animal?

DR. WILLIAMSON: I guess we wanted to select a nonhuman primate model. We have available to us the marmoset. We are doing a little bit of work in the marmoset, the small nonhuman primate

model, but that is slightly behind what we have done in the cynomolgus macaque, and really, it was in terms of the previous literature and the availability to us as cynomolgus macaque.

DR. QUINN: Thanks again, Diane.

[Applause.]

Our final speaker in this session Dr. Sue Welkos, Senior Scientist, Bacteriology Division, USAMRIID, Fort Detrick. Sue will be presenting on assays to establish correlates of protection.

Assays That Can Be Used To Establish Correlates of Protection

Dr. Susan Welkos

DR. WELKOS: We have been interested at USAMRIID in developing in vitro assays which might be predictive of immunity to plague in immunized individuals, and most of the focus of these developments has been utilizing the F1 capsule antigen and the V antigen.

The reason behind these decisions, of course, is quite clear by now, and I won't spend any time on it, but many early studies in animals

indicated that both of these antigens are highly immunogenic and highly protective.

Just, for instance, any combination of vaccines, we tried in a model, murine model, immunized subcutaneously and then challenged with Y. pestis strains CO92 compared to the old Greer vaccine, subunit vaccines containing either V11F1 or even better EF1 fusion construct that was made at USAMRIID. All of these provide significant protection and elicited high titers of circulating antibodies.

So, the question then became, can an immunological response to these two antigens be developed, such that it can be developed into an in vitro assay, which would then predict immunity.

Most of the talk today focused on assays we have been working on that mainly deal with the V antigen and the response to V, however, I wanted to spend a few minutes on a recently developed competitive inhibition ELISA based on anti-F1 responses that has been fairly successful and fairly well developed.

Many people contributed to the development of this assay including Drs. Evanovich, Tran Chanh, Dr. Andrews, Dr. George Anderson. In any event, you have heard this before, the basic outline of the competitive assay utilizes plates that are coated with the antigen, F1 here, and then dilutions of standard known anti-F1 monoclonal prepared, standard inhibition binding curve, and then unknown serum samples are similarly diluted, to each is added a competing antibody labeled biotinylated anti-F1 monoclonal antibody in this case.

Then, the plate is incubated and developed with a rabid anti-mouse stripped out of it, and conjugate. The bottom line of this assay is that in tests done with serum from mice that have been immunized with F1, there has been a very good correlation between the levels of competing F1 antibodies in this situation and protective immunity.

This just gives a summary of one study done with 163 mice that were immunized and then

challenged subcutaneously. It is plotted such that there were several different dose groups of animals that received the vaccine ranging from 0.1 to more than 10 micrograms.

This gives their level of competitive ELISA anti-F1 antibody. You can see that the nonsurvivors are shown in pink, purple and pink, those individual quantities circulating F1 specific antibody, and in blue are the survivors, and if the means of these two groups are calculated--it is not shown here--but the mean of the nonsurvivors was 11 micrograms of antibody per ml as compared to 86 micrograms per ml, for the survivors, and this was highly or statistically significant and correlated very well with protection.

Perhaps more interestingly, effective dose of 50 and 95 calculations, values were determined, and, for instance, it was determined that a circulating quantity of 420 micrograms of the antibody, 420 micrograms per ml provided effective protection to a 95 percent level.

So, ultimately, the goal, of course, would

be to find a similar kind of level in vaccinated humans.

The problem, of course, with this type of assay is it doesn't account for strains of Y. pestis that are F-1 negative, yet retain nearly full virulence, and have been shown to overcome F-1-based immunity.

As a consequence of this, there are several in vitro correlates of immunity to both F-1 positive and F-1 negative Y. pestis strains are in the process of being examined and developed.

As the alternate non-F-1 antigen selected, of course, V was our first choice, as has been mentioned over and over again at this point. It is an essential virulence factor, it is highly immunogenic, and can confer protection, anti-V antibody can confer protection by passive vaccination and the antigen by active immunization.

This is a diagram based mainly on one of the protective monoclonal antibodies applied by Jim Hill at DSTL and mentioned and discussed by Dr. Williamson, but in any event, Jim Hill developed a

set of monoclonal antibodies with different epitope specificities that were specific for different parts of this 326 amino acid V molecule, and whereas, antibodies directed towards more the N-terminus were found to not be protective in a mouse challenge model.

The passive immunization with these monoclonals did not protect, whereas passive immunization with various ones directed in the region of about 135 or 275 amino acid in that region, such as the 7.3 were found to be protective.

So, these kind of responses would be those that it weren't taken into consideration in developing an in vitro correlate.

As Dr. Williamson mentioned, both USAMRIID and DSTL have been working on competitive ELISAs based around a protective monoclonal antibody directed against V, and in this case, as she mentioned, they have been working with the 7.3.

This is just a very, sort of gross oversimplification of a couple studies, very nice

studies of Dr. Garmody--I might have pronounced that wrong--and Dr. Williamson and coworkers that came out recently in Vaccine where a competitive anti-V ELISA was described, and it just involves a couple studies that were done with an attenuated Salmonella live vaccine that produced recombinant V, and a DNA vaccine plus a booster protein of V, and both studies show that they could elicit partial protection with these vaccines and provided a nice range of sera for being able to use to develop in vitro assay to predict survival or not in ultimately challenged animals.

They had both assays for direct endpoint ELISA titers measuring V antigens specific antibody and a competitive ELISA based on competition of the serum antibody with this protective monoclonal.

However, there was no significant association reported between the titer of the competitive anti-V antibody and survival of these mice. There could be a number of reasons, but anyway we can discuss that later.

So, overall, this has been somewhat of a

challenge to develop solid in vitro correlates, but in the same vein, a competitive ELISA based on competition of a serum antibody with a protective monoclonal anti-V antibody has been worked on at USAMRIID namely by Tran Chanh and coworkers at USAMRIID, and a number of monoclonals directed against V have been made available to these workers, and so far they have identified 5 that produced high ELISA titers of antibody in vitro and also provided protection against lethal challenge of mice in vivo.

These 5 antibodies, well, I show them here, and as I mentioned, they exhibit high anti-V antibody titers in an endpoint ELISA and they can passively protect mice.

This is just a summary of some of the passive experiments. This is the summary of all 5 monoclonals, but basically, they provided approximately 50 percent to two-thirds protection of the animals and positive control gave total protection is rabbit, polyclonal anti-V antibody showed previously to be very protective, whereas,

untreated animals weren't protected.

I failed to mention the model here was immunization, treatment intraperitoneally with the antibody, and then challenged was with 25 LD50 by the aerosol route, so it was a fairly realistic challenge, and as I mentioned, the passive therapy protected against that.

I think that antibody was given 24 hours prior to challenge. I am not positive, but I think that is correct.

One of those antibodies, 141 was selected for use to develop an in vitro competition assay. It is not too interesting.

Also, I am not going to discuss this in detail, just to mention the obvious question, if these antibodies are protective, what is the epitope that they are recognizing. Dr. Chanh and his coworkers are just in the process of examining this question. They are using a protease protection type of assay, but beyond that I can't say too much yet, just to answer the question that is obvious.

I don't need to spend time on that, but it is the same kind of drill here. The plates are in this competition V-based assay. Plates are coated with V, a titer of protective monoclonal antibody is established that will give a sensitive level of detection of whole antibody, and then the samples are diluted out, the standard curve monoclonal antibodies diluted out, and the competing biotinylated monoclonal is added, and so forth, the plate is developed.

So, that was the development of the assay. Now, the investigators are, of course, in the midst of real contesting of this assay with sera from animals that have been immunized with F1-V and subsequently challenged. They collect the pre-challenged sera and assess levels of competitive anti-V antibody, and then correlate that with the ultimate survival.

The only thing interesting about this, this just shows one of the sets of sera that they have examined. These were mice that were immunized subcutaneously, two doses of F1-V, the fusion F1-V

antigen, and then they were challenged subcutaneously with 5 times 10^7 LD50 doses of the CU92 strain.

The four dose groups tested are shown here. This is the dose of the F1-V fusion vaccine, and this just gives the numbers of animals that we were working with.

This is a summary of the results. I will just show you this first. These are the sera from all the survivors, you know, pooled from all those groups, are assessed, and then, similarly, the pre-challenge sera of the nonsurvivors were measured in this assay.

It was found that the mean value of the survivors in terms of again the quantitative level of competing anti-V antibody is 44.6 micrograms per ml as compared to 7.8 micrograms per ml in the nonsurvivors, and this was highly statistically significant and are correlated very well with survival, and gave a predicted effective dose, 50 of 8.2 micrograms per ml of that antibody in serum.

I won't spend too much time now. The

obvious tests there are to perform the fact they are using sera of immunized nonhuman primates, this is in process. We are testing sera generously provided by Dr. Pitt and her coworkers involving the models of the African Greens and the cynomolgus macaques.

The first set of sera, most of the animals in the experiments either lived or died, and the sera aren't appropriate really for trying to assess a correlation between survivors and nonsurvivors, if you have everybody has lived or everybody has died makes it kind of difficult, but more experiments have been done, more sera has been collected, and Dr. Chanh and his workers are very busily assessing the sera.

I can't say a lot about it yet unfortunately, however, I took the data that they did do, they did assay from some of the very early studies where all the animals in one group died and all the animals in the others lived, and I kind of pooled it together.

They took the competing ELISA titers of

these four groups of survivors and nonsurvivors, and it does appear that we are getting a similar trend in the nonhuman primates that we saw with the mice in that the survivors will indeed have a significantly enhanced level of competing anti-V antibody compared to the nonsurvivors.

Now, in addition to the antibody-based assay, we have been looking at sort of a more functional assay of anti-V activity to provide an additional correlate, in vitro correlate, and we have been examining assays for antibody based on neutralization of macrophage cytotoxicity.

As nicely described by Dr. Bliska, at least in the later stages of infection with a virulent Y. pestis, the organisms in vivo resist phagocytosis and they cause an infection that is mainly extracellular.

In vitro, this can be modeled by appropriate pregrowth of Yersinia pestis will put them in a state that they resist phagocytosis and are cytotoxic for macrophages. We wanted to see if we could develop this model as the basis of an

additional cell-based in vitro correlate.

So, the question was can antibodies that protect against pestis in vivo neutralize this in vitro macrophage cytotoxicity assay, and, if so, what is the role of V and anti-V in all of this, and as you have heard and I won't belabor the point, V is required for the type III secretion-mediated translocation of the cytotoxic Yops.

This is just a nice, very simple diagram that was published in an article in 1999 by Drs. Field and Straley, and this just shows the close contact that is required for this process of the pestis inducing the cytotoxicity of macrophage, direct contact between the organism and cell stimulates the production of the Yops and their secretion and translocation into the target cell.

As you can see, what has been called sometimes the injectozone, which is the needle through which the Yops are translocated, it appears that V is the special component of this, so it is essential in the actual delivery of the cytotoxic Yops.

V, of course, has multiple roles and I won't discuss any of the rest of this further, but if V is so essential in the translocation of the cytotoxic Yops, the question was can antibody prevent the whole cytotoxicity.

We tested this. Steve Weeks was the postdoc in my lab, and he developed a nice macrophage assay to examine these questions, and the initial assays were done just simply looking at LD8 release, a terminal marker of necrosis, cytotoxicity, and cell death, and he found that when macrophages were grown, well, when Yersinia pestis was grown in vitro for 2 hours at 37 degrees, and then incubated, pretreated or incubated, the cells were incubated with normal rabbit serum, and these organisms were then used to infect cell cultures, there was no effect of the normal serum on the cytotoxic activity, that indeed the Y. pestis was cytotoxic for the macrophages and killed them, however, the organisms were similarly pregrown in vitro and then incubated with the rabbit anti-V antibody.

Then, the mixture then used to infect macrophages, that this treatment seemed to ablate the cytotoxicity. The same effects were seen with the isolated FAB fragments of the antibody, suggesting that the protection was just not merely due to recognition by the FC portion of the FC receptors of the cell.

We wanted to know if the death of the macrophages was due to necrosis or perhaps might be a reflection of an apoptotic or programmed cell death phenomenon, so we, instead of measuring LDH or besides measuring LDH release, we also did assays to measure the caspase enzymes. Caspase enzymes are proteases that are made specifically only during apoptosis, programmed cell death, and the caspase-3 enzyme is one that is made early in the process of the cell going through this death phase.

We wanted to see if this marker could correlate with what we have seen with the LDH release, and basically it did. When you pregrew the bacteria, the pestis, the fully virulent

organisms or the pgm-minus organism, the same thing, they effectively cause the increased production of greater levels of caspase than is seen in uninfected cultures, so it seems to induce an apoptotic type death pathway.

As expected, uninfected cultures--I didn't show this--cultures infected with the organism that was cured on its virulence, also when there was no cytotoxicity, and organisms with a mutation in a critical translocation protein YopD also were ineffective.

After developing the assay, we wanted to test whether it was predictive in these animal studies. The question was: Can serum macrophage cytotoxicity neutralizing activity from immunized animals serve as a quantifiable predictor of protective immunity?

The first tests were done with mice immunized with F1-V, similar to the set that I mentioned for the competitive ELISA. These animals received 2 doses subcutaneously with different doses of F1-V fusion vaccine, and then were

challenged subq, and we tested the association between survival and cytotoxicity neutralizing activity of the antibody and also the effect of the vaccine dose.

This just gives the results, sera from individual mice. The mice were immunized. During the course of immunization, from day zero up to just before challenge, sera were collected from the animals to see if there was sort of a development of neutralizing antibody or development of the anti-V antibody.

We took all these sera from each mouse and titrated each of them, tested different dilutions, and then used the sera in the in vitro assay to incubate with the organisms prior to the infection of the macrophage cultures with the organisms.

This shows the data for one mouse that ultimately lived after immunization. As you can see, over time, from day zero to just prior to challenge, there was an increasing development of antibody that was better with time able to neutralize macrophage cytotoxicity as indicated by

the quantity of caspase enzyme that was detected, so that quantity of that death-related enzyme was dropped with time as more antibody--the data is not shown here, but also the direct ELISA titers of the anti-V antibody increased with time.

As they increased with time, the amount of neutralizing activity also did. It just shows two different dilutions. In contrast, these are the set of sera from an animal that ultimately died, and as you can see, there is no real pattern to the development, no real evidence that cytotoxicity neutralizing activity has developed.

We submitted the results of the studies of all the animals and all these titrations for statistical analysis, and we found the statistical outcome was that the vaccine dose together with the decrease in serum caspase from days 1 to 56, just prior to challenge, correlated well with survival.

This is sort of shown graphically here and that the change in caspase levels over time during the process of immunization is plotted at the bottom. Negative values mean that there is

increasing neutralization of the cytotoxicity, which means decreasing levels of the caspase. Zero or positive values indicate that there is no effective neutralization, and you can see this represents all the mice from the 0.1 microgram dose group of F1-V.

This gives the probability of survival from zero to 100 percent. In animals that were shown later to survive, they had negative values in that they showed a large drop in caspase levels during the course of immunization whereas the animals that ultimately died did fail to develop neutralizing antibody.

We did similar studies with animals that were just vaccinated with a single dose of F1-V. This just shows the groups that we had. There were 7 vaccine dose groups. They all received one dose of vaccine from 30 micrograms to zero on day zero, and then challenged on day 28.

This gives the summary of the results. Again, animals immunized with one dose, we found that the mean cytotoxicity-neutralizing value of

the sera of the survivors was highly significantly greater than that of the nonsurvivors and quite predictive of protection.

This was the first set of reagents we have been able to test where the macrophage cytotoxicity assay by itself was a marker predictive of detection. It wasn't dependent on vaccine dose or anything. It was independently predictive of infection.

Remaining challenges. Of course, we want to kind of verify the usefulness of this assay using sera from primates that have been immunized and we are, as I mentioned, in the process of trying to analyze such sera now.

The ultimate goal would be to determine a level of serum in vitro neutralizing activity that predicts protection in both encapsulated and nonencapsulated organisms.

So, just to summarize, promising correlate assays of F1 and V antibody activities are being developed, however, a thoroughly tested correlate assay for immunity to plague has yet to be defined.

This will require for both the competitive ELISAs again rigorous tests with sera from nonhuman primates and the same requirements for macrophage cytotoxicity assay. Decide on a very definitive and rugged standardized assay and then complete tests with nonhuman primates.

I won't go into this, but in the event the macrophage caspase-based enzymes fail to provide a very good correlate of immunity, we are also at the same time examining other markers of cytotoxicity that cover the whole range of the apoptosis cascade from very early events in apoptosis to the terminal necrosis.

We are in the process of looking at a number of different assays plus we are also, in addition to mouse cells as a macrophage cell type, we are looking, examining whether human-derived cells might be more better predictive, their responses might be more predictive, for instance, of the activity you would get with the nonhuman primate sera, so we are sort of actively looking at this.

There have been contributors over the years to this project. Tran Chanh, of course, contributed some data to this presentation, and he and Sylvia have provided numerous mouse sera, as well as monoclonal antibodies.

Steve Weeks, a postdoc in my lab, was the first to develop the macrophage in vitro assays we had. Jim Hill provides monoclonal antibodies, such as the 7.3. Jackie Bashaw is currently working very hard on these assays in my lab. Kelly Rea has been previously associated with that work, and then a number of people have contributed animal sera from their vaccine studies, Jeff Adamovicz, Gerry Andrews, Louise, Chris Bolt.

That's it, the end. [Applause.]

DR. QUINN: We do have some time for questions.

DR. MIZEL: Steve Mizel, Wake Forest.

What form did you immunize with F1-V, did you have alhydrogel?

DR. WELKOS: I believe it was always formulated in alhydrogel.

DR. MIZEL: I have another question which relates to testing in these animal models. We are making up antigens that are really not done in a GMP facility. So, is it possible, do you check for endotoxin levels and bacterial DNA, things like that, so that when we transition to humans at some point that we are not--and we are making fewer preparations that can go into humans, that we might see different results?

DR. WELKOS: I believe endotoxin levels have been checked. DNA, yes, in some instances, because there are studies that are kind of pre-GLP at this point in time, can anybody else from my place comment on that?

I can't give you numbers, but these kind of tests are being done because some of this work is at nearly GLP stage.

DR. WILLIAMSON: I just wonder whether you can say anything about how these assays read out between mouse and nonhuman primate, are you getting very similar results in the nonhuman primates?

DR. WELKOS: Are you talking about the

competitive?

DR. WILLIAMSON: Yes.

DR. WELKOS: Just that one graph I showed that suggested that it was a promising indicator that the primates that went on to survive were giving higher titers of competing anti-V antibody than the nonsurvivors, but like I said, we are just now collecting some data which provide a nice range of sera from survivors and nonsurvivors, which we have been needing, and they are being tested, but it seems promising, but beyond that, I can't say.

It will be very nice when they have characterized the epitopes, the specificity of some of these protective monoclonals, and more can also be said at that point I think.

DR. WILLIAMSON: Another quick question is then do you see a difference between the African Green and the cynomolgus model in the competitive ELISA?

DR. WELKOS: I don't know. I don't have that information.

DR. BLISKA: My questions are about the

cytotoxicity assay. It looks like it's working great. I was curious about a couple of details.

When you are using serum, have you ruled out that there is complement-mediated killing of the bacteria during the cytotoxicity stage, for example?

DR. WELKOS: Not directly.

DR. BLISKA: I think the organisms are resistant, but I was just curious.

DR. WELKOS: No, I am sorry, we haven't directly addressed that question that I can think of.

DR. BLISKA: The other issue was I have noticed--and maybe you have switched to using Yersinia pseudotuberculosis--and I noticed that Dr. Williamson had also. I am wondering, is there a reason for that, is it just more reproducible?

DR. WELKOS: It works also with pestis, so we can do it under BL2 conditions. We use a straight test, pgm-minus and Pla-minus, highly attenuated, but Jim Hill clued us in to the pseudotuberculosis, the strain that is mutated for

its own V and is transformed with this nice expression plasmid to PTRCB plasmid, that I think Dr. Forrestburg originally isolated, that produces a nice quantity of V, and it just gives nice, cleaner results. It gives better cytotoxicity sometimes in our controls. We always have a set of controls, you know, untreated to make sure that we are killing the cells.

They seem to give comparable results with the pestis, but we have just gone with the Y.ptb for now just because it is easier to handle, you know, better, easier, cleaner results.

DR. BLISKA: The last issue is you mentioned that sometimes in this, I would say you get some translocation of the Yops even with neutralizing antibodies, so I was just curious if you considered measuring cytokine productions for something in addition to apoptosis, it might be another reflection of a neutralizing.

DR. WELKOS: That would be an excellent thing to do. The only thing we had done was try to see if anti-V antibody would kind of neutralize the

stimulation of IL-10, and that was kind of a bust, but your suggestion is well taken. That is something we should consider.

DR. QUINN: Last question.

DR. PERRY: Bob Perry, University of Kentucky.

Just to quickly answer Jim's questions about complement-mediated killing, they are resistant in the absence of specific antibody, and I think Bob Brubaker had a paper that showed that it was probably due to the short LPS, no antigen side chain was involved in that.

But in the absence of a specific antibody, they are resistant, so it is not a problem with the assay.

DR. QUINN: Very good. If there are no more questions, then, we will close the session and thank the speakers once more for their presentations.

[Applause.]

[Luncheon recess taken at 12:20 p.m.]

AFTERNOON SESSION

[1:40 p.m.]

DR. MEYSICK: We will get started for the next session.

Session 3: Human Disease and Relevant Animal Models

Moderator: Dr. C. Richard Lyons

The next session is Human Disease and Relevant Animal Models, and the moderator for this session is Dr. Rick Lyons from the University of New Mexico.

Rick.

DR. LYONS: Thanks, Karen.

This session, we will take a look at the epidemiology of human disease and how the animal models relate to that.

The first speaker is Jacob Kool from CDC, Fort Collins, and he will be talking on plague epidemiology and human disease.

Plague Epidemiology and Human Disease

Dr. Jacob Kool

DR. KOOL: I would like to thank the organizing committee for inviting me to this very

interesting workshop.

I will be the one I guess giving the background talk about clinical aspects of the disease and epidemiology, but I am especially excited about the opportunity to tell you about the CDC clinical trials that we are currently doing. We are evaluating drugs and diagnostics in Madagascar and in Uganda. I wonder if those drugs might also be used for a vaccine trial.

I developed a slight cough on my way back from Madagascar, I just came back a few days ago. I hope it is not a slight case of pneumonic plague, but I have to apologize because I didn't have time to submit my handouts in time to be included in your handout.

In this presentation, I will talk about the epidemiology of plague in the world, in the United States, and the implications of bioterrorism. I will talk about clinical aspects of plague, of course, naturally occurring plague with an emphasis on pneumonic plague.

Karen Meysick suggested I should bring a

lot of x-rays. Now, it is pretty hard to get x-rays of pneumonic plague, but I have done my best. At the end, I would like to mention a few field sites in Uganda and Madagascar.

You have already seen this picture, the global distribution of plague. The red areas are actually the interesting areas where we think there are still sylvatic, endemic foci of plague. It is the western U.S. where there are only about one or two cases a year now.

South America, Asia, there are probably still a lot of cases in southern China, we don't hear a lot about them. They don't always get reported. In fact, more than half of all cases, about 80 percent of all cases are reported from this area, eastern Africa and Madagascar. Up to a few years ago, Madagascar reported about 50 percent of all cases of plague in the world through WHO.

In the United States, as you all know, plague was first imported into the U.S. in 1899. It first caused outbreaks in San Francisco in the Bay area, and in Los Angeles in '24, and then it

suddenly seemed to almost disappear until it shot back up in the sixties and especially peaked in the mid-eighties.

Maybe in this period in between, these outbreaks usually were transmitted or propagated by urban rats, and these outbreaks in these cases are usually associated with wild rodents in the western plains in U.S., so perhaps these rodents needed these years here to get infected, to establish the infection.

So, nowadays, most cases of plague occur in New Mexico, and they are usually sporadic cases of bubonic plague associated with rodents like prairie dogs.

This is the way plague is transmitted. Our plague ecologist gave this slide to me. Here is a picture of a prairie dog. There is this epizootic cycle of prairie dogs and their fleas, and occasionally, very rarely really, it gets transmitted to humans, for example, when a human passes a prairie dog colony, and the dogs, when they are dead, the fleas will look for another

host.

What happens a lot, too, is that other animals, for example, cats who are hunting for prairie dogs get infected, and cats can develop plague especially pneumonic plague. The only cases of pneumonic plague that we see nowadays are usually cat associated.

So, when the case of bubonic plague turns pneumonic, then, you can have the cycle of transmission among persons, of course, and these domesticated animals and mice and rats theoretically can also sustain a cycle, but this is very rare nowadays.

This is a typical picture. In 2002, there were two cases, a couple who traveled from their home, this home in New Mexico to New York City and developed plague while they were in New York City. You can see that this is a typical habitat of prairie dogs, and there is clearly a short interface between humans and wild rodents in this type of dwelling.

Plague occurs mostly in the summer in the

United States. This is the clinical presentation that we see in the United States. Over 80 percent is bubonic plague. Then, the next chunk is septicemic plague, and only about 2 percent are called pneumonic. This is the primary presentation. Of course, there are some pneumonic cases among those bubonic plague cases, secondary pneumonic cases.

The bioweapon potential of plague, as we mentioned before, it is thought that Yersinia pestis was recognized by the former Soviet Union for aerosol delivery. Theoretically, it can be engineered for antimicrobial resistance or virulence. F1 deficiency, I am told is quite easy to get into the bacteria, and this would have implications for vaccine development, but also for diagnostics. Most of our diagnostics are based on detecting the F1 antigen.

Theoretically, maybe lyophilized formulations could be used as a weapon. We don't know what will happen in the environment if after releasing over a city, if it will establish itself

among the urban population again.

This is the only Category A bioterrorist agent that can also be transmitted from one person to another.

So, in 1970, WHO called in an expert panel, and they estimated that if 50 kilograms of Yersinia pestis would be released over a city of about 5 million, this could cause about 150,000 cases, more than 30,000 deaths, hospitalization for up to 100,000 people to a secondary spread, they thought might affect another half million people with up to 100,000 deaths.

This is an old picture of typical bubonic plague, typical bubo. This must be the place where they tested the aspirate, where the blood is.

Here is a picture of a septicemic plague case. All you can show really is a very sick, obtunded patient.

Pneumonic plague. As we mentioned before, there are two forms of pneumonic plague. Secondary pneumonic plague is what we see normally. It is caused by hematogenous spread of the bacteria from

a bubo or from blood in the case of septicemic plague to the lungs.

Primary pneumonic plague, of course, is caused by direct infection of the lungs, and this is the disease that we are really interested in today, because that is caused by terrorism, as well.

Primary pneumonic plague, what we know about primary pneumonic plague is mostly from historical accounts especially the large outbreaks in Manchuria in 1910 and 1920 where, in total, about 76,000 people died of primary pneumonic plague. Since then, there have been only very small outbreaks.

We know that it has a very short incubation time, probably 2 to 4 days, and the range may be between 1 and 6 days, but there are a lot of questions about those historical accounts, about determining the date of onset with those patients.

It typically has an acute fulminating course characterized by a systemic inflammatory

response syndrome with disseminated intravascular coagulation, ARDS, so they require intensive support, and just a few cases could easily overwhelm the capacity of the health care system.

Mortality is 100 percent. People usually die within 3 to 6 days after onset if they are not treated early, and that means it is clearly necessary to give the first dose of antibiotics within 20 hours of onset, and that is quite a challenge.

In the United States, as I mentioned, there have been some outbreaks of primary pneumonic plague. The only cases really of human-to-human transmission were in 1919 and in 1924 in Oakland and in San Francisco. Since 1925, there has been no human-to-human transmission of plague in the United States.

There have been 8 cases of primary pneumonic plague. Six of those were associated with cats. Most of them were veterinarians who were treating a cat with pneumonic plague. One was associated with a laboratory accident. Someone was

centrifuging Yersinia, and the vial broke, and one remains unknown.

I tried to give a description of what does primary pneumonic plague look like, and I have to go to very old sources, Wu Lien-Teh from Manchuria in 1926, Pollitzer, Tom Butler in Vietnam.

What they seem to describe as a typical case is an initial noninfectious stage which might last several hours, up to about 24 hours. Wu Lien-Teh calls it a noninfectious stage because he noticed that hardly any of these patients ever contaminated other people during this stage.

This stage is characterized by a sudden onset of malaise, chills, severe headache. There is increased respiratory and heart rates, and during this stage, the temperature rises steadily.

After several hours, you will see a dry cough develop which becomes progressively productive, but even the sputum still doesn't contain many plague bacilli. It was usually very hard to find any bacteria in the sputum.

This might continue for hours up to a few

days even, and in the final stage, this means a few hours before they die, maybe only one hour before they die, the patient will have bright red sputum, and if you look at that under the microscope, you find many plague bacilli in almost pure culture, as they describe it. These are the patients that are very infectious.

So, it is kind of hard to recognize a case of pneumonic plague in the early stages, and in these days, the patients should rarely actually progress to this stage here. This is only when patients are not treated with antibiotics.

Here are some pictures from the outbreaks in Manchuria. Here are two cases of pneumonic plague. This is a patient in the early stage, and this is a patient in the final stage, just before death I guess.

Here you see a patient with blood-stained bed linen who is coughing up red sputum. Here is one household, everybody, all the dead people in one household. There were lots of pictures like this.

Here are some more pictures of pneumonic cases. You see how the health care workers protected themselves with masks. These patients were examined in the open air.

The only picture that I could find, the only x-ray of the primary pneumonic plague case in the United States is this one. This was a 22-year-old male in California. I believe it was the mid-eighties. It is not clear where he got his infection, but he started to feel ill on a Friday, and he even reported to work on Monday, and only on day 5, on Wednesday, is when he was brought into the hospital moribund, he was very ill, in severe respiratory distress.

This is the x-ray that was made then. You can see a large infiltrate in the right lung. Only at 12 hours later, the patient looks like this. He developed adult respiratory distress syndrome, and he died within two days of hospitalization in spite of mechanical ventilation.

Here is his hand. This was before we gave this disease the connotation of the Black Death.

This necrosis occurs, not just in the hands and feet, but in all organs in the body.

Here is another. I apologize for the quality of this picture. There is an interesting article by Alsifom in 1981, but all these cases are secondary pneumonic cases.

You see, in this case, a large number of deaths. Only 12 hours later, this has become much worse with bilateral infiltrates, diffuse bilateral infiltrates.

This patient, you see left pleural effusion, and this was actually made a few weeks after recovery. He still has a cavitary lesion.

This patient shows bilateral pulmonary parenchymal infiltrates.

This is a case of bubonic plague that does not have pneumonia. This whole picture is actually caused by the DIC, not by infection of the lungs. So, an x-ray doesn't always tell you if it is pneumonic plague or not.

Treatment of plague is parenteral. It is done with these antibiotics - streptomycin,

gentamicin, doxycycline, ciprofloxacin. Gentamicin and ciprofloxacin have not been FDA approved although they are part of the national pharmaceutical stockpile. Prophylaxis can be done with co-trimoxazole.

Person-to-person transmission. Contrary to what many people believe, plague is not very contagious. The risk is not as big as people think. The last time this happened in the U.S., as I said, was 1924, and it only happens in very close contacts. You have to be closer than at least 2 meters, and the surgical mask is probably protective. This is what health care workers in Manchuria used, and it was quite effective. It was made of cotton.

Like I said, they are only infective in the later stages, and after one day of antimicrobial therapy, patients are not infectious anymore.

So, I would like to show you a bit of our field sites in Africa. We are doing field sites in Uganda and in Madagascar. Our project consists of

two parts. One is to determine safety and effectiveness of gentamicin. In Uganda, we compare it to doxycycline. In Madagascar, we compare it to streptomycin because those are the nationally used regimens.

We also take this opportunity to evaluate newly developed rapid diagnostic tests, dipstick kind of tests. My colleague, Marty Schriefer is here. If you have any questions about this part, he will be happy to answer it.

We are evaluating four brands of dipsticks, and they are all based on detecting the F1 antigen. This study, by the way, is funded by FDA/CDER.

These are the four diagnostic tests with dipsticks. All of them were originally developed by the U.S. military, but this one was taken over by the Institut Pasteur in Madagascar, and they are already using it in that country. These three are newly developed, and we are evaluating those together with the Institut Pasteur dipstick.

For now, they have only been approved for

nonhuman use. We hope to change that. They are showing very good results so far.

These are the countries - Uganda and Madagascar, and we found women to collaborate with us on this clinical trial. We have been preparing for this study for about two years now. We had to completely renovate and equip central laboratories in each country that were close to the plague endemic areas.

The field sites where patients actually come to the clinic had to be equipped also with colorimeters to test kidney function, and we did all kinds of other things, electricity, refrigerators, communications. We had to get vehicles to transport specimens and to transport patients, and we have hired and trained many field staff. IRB approvals were quite a challenge, but we got it approved. Accounting is also important in these countries. Uganda ranks I think number 5 among the most corrupt countries in the world.

In Uganda, plague tends to occur in these highlands here, at the border with the Congo in

northwestern Uganda. This is the West Nile region.

Our field sites. We have our central laboratory located in Arua, the largest town of the West Nile region, and we have 14 field sites along the Congo border where we expect to see cases of plague.

Uganda sees about between 200 and 500 cases per year in that small area. Cases have a seasonality. They occur mostly between September and December. This is after the harvest when people bring their harvest into the house, and the rats follow the harvest, and they bring plague into the house.

Here are some pictures of rural clinics in Uganda. I really wanted to put your attention to this one. The plague isolation ward of Agiermach.

This is a plague case. A young boy who had bubonic plague last year.

This is the laboratory. It is an area that had been ravaged by civil war several times, the last time about 15 years ago, this building was sacked. We were donated this building, and we

renovated the whole thing, and it now looks like this.

This is when our equipment arrived. They brought it in a big container. There is no crane, so they had to tie a rope to a tree and then drive the truck out underneath it. This just gives you idea of the remoteness. Fortunately, they had taken out the equipment before they tried this, so this is what happened.

We were lucky. This is Marty Schriefer. He is sitting right there. We were lucky that he was standing on this side, but no problem, we just roll it back, and it is now our storage shack.

I will show you some pictures of Madagascar. This is rural Madagascar. They have not yet invented the chimney. The reason why in Madagascar there still are small outbreaks of pneumonic plague, family outbreaks of pneumonic plague, at night they hermetically close their doors and windows. I think it has something to do with a fear of ghosts, maybe also to keep the warmth in. As you see, there are no chimneys, so

it is really a great place to get any respiratory infection.

Suspected cases of plague in Madagascar, as reported to WHO, they have reported up to 3,000 cases in the mid-nineties, but this has gone down, and confirmed cases have always been quite low. Because of the distances, it is really hard to confirm any cases microbiologically.

The cases that I have gone down for, because they have started to use the dipstick test, and they were able to show that many of these cases actually were not plague. So, now there are several hundred cases a year in Madagascar. Lethality is now about 20 percent.

Here is a typical bubonic plague, an early bubo without pus. They regularly see pneumonic cases, like I said, but these are already recovering or recovered almost.

Our field sites are in the highlands. This red area is where plague occurs in the highlands mostly. We have chosen this small area where the incidence has been the greatest in the

last five years, and we have equipped 10 rural clinics and the Central Plague Hospital in the capital of Atonaria [ph].

This is the Central Plague Hospital. We built our lab there completed by a safety cabinet and freezer and everything. The rural clinics have been equipped with solar panels. Here, you can see someone working with a centrifuge that we gave him on the first samples from the first case of this season.

Here is a colorimeter that he is going to use to determine creatinine for renal function.

This is our first case. She was brought in severely dehydrated after a ride over this road actually for six hours in a wheelbarrow. This is the type of isolation that these people live in. This is actually her village. We went out there two days ago. This was e-mailed to me last night. It is only a hamlet of about five of six houses.

The dipstick test did very well on this patient. I haven't heard how the patient is doing now, but we expect that she will recover.

This is my last slide. So, our timeline for our clinical trials are to go--we have just started, like I said, this October--we will go for two seasons. So, the project is projected to end in the spring of 2006. After that, we think it will be a waste not to keep on using these laboratories in these field sites, so we hope that we can find a way to continue this work. Maybe we can develop novel methods for control among rodents and fleas. Maybe we can follow up our clinical trial to test fluoroquinolone, and who knows, maybe these sites are useful for vaccine evaluation, as well.

Thank you for your attention.

[Applause.]

DR. LYONS: In order to stay on schedule, we probably have time for one or two questions.

DR. FROTHINGHAM: Rich Frothingham, Duke University.

You alluded to the importation of plague to the Americas in San Francisco, I think in the latter part of the 19th century. I wanted to pose

to you the question that I get all the time, which is, of course, this would never happen, my mice are never going to get loose, but the question is if my mice get loose, they will be consumed by some predator pretty quickly, what is to stop the development of a new sylvatic focus, and why is it that these foci develop in certain places.

DR. KOOL: That is a very good question. I don't think I have an absolute answer to that, because at the same time when these rats were introduced into San Francisco Harbor, of course, they came to New York, as well, and to Houston, everywhere where there is a port.

So, there must have been something about the western U.S. that is more friendly to plague, to developing a sylvatic focus. Of course, in those days, hygiene was much worse, there was much more interface between humans and rodents in their houses, so, yes, the big question is, could it establish itself again among the urban rodent population. I don't know the answer to that.

DR. McINNES: Pamela McInnes, NIAID.

I am sorry, Jacob, if you mentioned this, I didn't get the actual design of your clinical trial, your interventions. I didn't get that.

DR. KOOL: I didn't have much time to go into that in detail, but what we do is we compared treatment with gentamicin to the treatment that is normally used in those countries, to the national standard, approved standard of care.

In Uganda, the approved standard of care is doxycycline, or tetracyclines in general, and in Madagascar, they still use streptomycin, intramuscular injections. So, a patient with plague who comes in with suspected plague is randomized into one of two treatment arms. They either receive gentamicin for 7 days or they receive the other drug, the national drug, and then we follow them equally.

DR. LYONS: I just have one question. If anybody in the crowd had a diagnostic, however, are these sera at all available, or are you going to stockpile them, so that people could have access to the infected sera to test against gold standards?

DR. KOOL: Yes, we thought this was the great opportunity to get new specimens from plague patients, so we do ask for the patient's consent to keep their serum and their aspirates, and we plan to transport them back to the United States.

DR. LYONS: Great.

Thank you very much.

[Applause.]

DR. LYONS: The next speaker will be Pat Worsham from USAMRIID on small animal models of plague.

Pat.

Small Animal Models of Plague

Dr. Patricia Worsham

DR. WORSHAM: I was asked to concentrate today on historical perspective on small animal models for plague and to try to tie that in with the animal models that we are using predominantly today.

One of the very first animal modelers was Yersin himself. He isolated a live attenuated vaccine strain which he found was virulent in rats,

but not in five species of macaques.

He was quite interested in determining which species most closely reflected that of the human, so he identified a human volunteer, in this case himself, and he injected himself, not like Haffkine with a killed vaccine, but with a live vaccine that he hoped was attenuated.

Luckily, he survived this, he had only transient fever, and he declared after this that he believed that the susceptibility of the macaque more closely resembled that of man than of the rat. Not something we can do in today's environment, but interesting nonetheless.

A number of small animal models have been explored. The most common are the mouse, the guinea pig, and to a lesser extent, the rat. Other models have included ground squirrels, rock squirrels, the multimammate mouse in South Africa, various other rodents, lagomorphs, and domestic cats, but there are inherent difficulties in comparing these models.

First of all, historically, there has been

very little consistency in the strains open for study and the strain of animal chosen for study, or the source that the animal was obtained from. In fact, some of these animals were obtained from the wild historically.

There is little consistency in the way the challenge inocula are prepared to the way that experiments are conducted, so it is very hard to compare experimental studies because of this.

The really consistent thing with all of these is that the predominant antigen that has been looked at over the years has been the F1 capsular antigen which you have already hear about today.

Perhaps the best characterized model is that of the mouse. It has certainly been the most utilized. It's an accepted model of bubonic and pneumonic plague. The pathology for the most part resembled that of human disease. It is desirable, obviously, in terms of handling, space, and expense. They are small, inexpensive animals.

You can have a lot of them, and it is a well-established model for both active and passive

immunization that has been alluded to by other people earlier. It is also useful for nontraditional or modern vaccine strategies.

The LD50 subq is from 1 to 10 CFU and, by aerosol, from 10^4 to 10^5. This is inhaled dose.

Also, the availability of different mouse strains and our knowledge of mouse genetics allows us to explore the role of various components of the immune system in both innate resistance and acquired immunity, and Diane has already alluded to that, as well.

In 1949, K.F. Meyer described the disease progression in mice challenged parenterally with Yersinia pestis, and it is not inconsistent with what we see in clinical cases of bubonic plague.

Fairly soon after exposure of the animal to the organism, they are carried to the regional lymph nodes, transferred to the thoracic duct and the bloodstream. This low grade bacteremia may go on for several hours, it seeds the liver, the spleen, and bone marrow in mice, and after replication of these organisms, there is a terminal

heavier bacteremia.

A number of methodologies have been used to look at what is hoped to be a model of pneumonic plague in the mouse. Small particle aerosols induce primary pneumonic plague. This has been shown pretty consistently.

In many cases, there have been aerosols used which give variable particle size. In some cases you get disease that is characterized, rather than primary pneumonic plague, it is characterized by cervical bubos and septicemia, so it is a different disease process caused by these larger particle aerosols. Pneumonic disease has also been reported in intranasal installation, but only about 10 percent of the inoculum actually reaches the lungs in this case.

There have been a lot of live attenuated vaccines evaluated in the mouse. The results are quite dependent on the type of attenuation that is present in the strains, but some good protection has been demonstrated. There is residual virulence in some vaccine strains, and this has been a